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Cutavirus DNA in malignant and non-malignant skin of cutaneous T-cell lymphoma and organ transplant patients but not of healthy adults

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40-word summary of the article's main point

This study revealed a significantly higher human cutavirus DNA prevalence in the skin of cutaneous T-cell lymphoma patients, compared to organ transplant recipients and healthy adults. This suggests that dermal CuV DNA is significantly associated with CTCL.

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Abstract

Background. Three new parvoviruses of *Protoparvovirus* genus, bufavirus (BuV), tusavirus (TuV) and cutavirus (CuV), have recently been discovered in diarrheal stools. CuV was further detected in a proportion of cutaneous T-cell lymphoma/mycosis fungoides (CTCL/MF) skin samples and in one melanoma.

Patients and methods. With novel multiplex quantitative PCR (qPCR) and antibody assays, we studied three patient groups for BuV, TuV and CuV DNA and IgG: CTCL patients, immunosuppressed solid-organ transplant recipients, and immunocompetent healthy adults.

Results. CuV DNA was detected in skin biopsies of 4/25 (16.0%) CTCL and 4/136 (2.9%) transplant patients, but not in any of 159 skin samples of 98 healthy adults. The dermal CuV-DNA prevalence was significantly higher in CTCL patients than in the other subjects. CuV DNA was further detected in healthy skin of four organ transplant recipients, two of whom also had CuV-positive skin carcinomas. One CTCL patient harbored CuV DNA in both malignant (CTCL, melanoma) and non-malignant skin and sentinel lymph nodes, but not in his prostate. The CuV IgG seroprevalences were among CTCL patients 9.5% (4/42), transplant recipients 6.5% (8/124), and healthy adults 3.8% (3/78). BuV and TuV DNAs were absent and antibodies infrequent in all cohorts. Parvoviral antibodies were shown to persist for ≥ 20 years and dermal CuV DNA for four years. All three CuV-DNA-positive patients, with both biopsies and sera available, were CuV-IgG positive.

Conclusion. Our results suggest that dermal CuV DNA carriage is associated with CTCL. Any putative roles of CuV in the carcinogenesis must be determined in forthcoming studies.

Introduction

Cutavirus (CuV) is the newest human parvovirus described [1]. It belongs to the *Protoparvovirus* genus together with two other newly discovered parvoviruses, bufavirus (BuV) and tusavirus (TuV), identified in 2012 and 2014 [2-4]. These three protoparvoviruses were all originally discovered in diarrheal feces, and CuV DNA was additionally identified by *in silico* analysis in pre-existing metagenomic libraries of skin biopsies from two cutaneous T-cell lymphoma (CTCL) patients. PCR studies further showed CuV DNA in the malignant lesions of 2/15 French CTCL patients, while 26 other skin manifestations and 3 healthy skin samples were CuV-DNA negative [1]. CTCL represents a group of malignancies of mature T cells, for which viral etiology has been long sought but not found [5]. CuV DNA was recently detected also in the cutaneous melanoma of a patient in Denmark [6]. Like other human parvoviruses, CuV induces a humoral immune response; however, with low seroprevalences (0-5.6%) in all countries analyzed [7]. Further studies of CuV-DNA occurrence in skin and other tissues as well as serological studies are needed to fully uncover the etiological role of this emerging virus in neoplastic and other human diseases.

In this study, we set up a multiplex quantitative PCR (qPCR) to detect and quantify BuV, TuV and CuV DNAs in altogether 355 healthy and diseased human skin from 25 CTCL patients, 136 organ-transplant recipients, and 98 healthy adults. Furthermore, we analysed 266 serum samples from 244 subjects with in-house BuV1-3-, TuV-, and CuV-specific IgG EIAs to assess the human protoparvovirus seroprevalences in these cohorts.

Materials and methods

Patients and Samples

Patients with cutaneous T-cell lymphoma

The CTCL patients were diagnosed, based on detailed dermatopathological and immunohistological examination, at the Department of Dermatology, Helsinki University Hospital [8]. Formalin-fixed and paraffin-embedded (FFPE) skin biopsies were studied of CTCL lesions from 25 CTCL patients, 23 with mycosis fungoides and two with leukemic Sezary syndrome (median age 64 years, range 32 – 89 years). From two patients with CuV DNA in the original skin biopsy, additional tissue biopsies were retrieved from the pathology archives. Serum samples were available of a cohort of 42 CTCL patients [9], including 2 of the CTCL patients whose skin biopsies were analyzed.

Transplant recipients

The immunosuppressed solid-organ transplant cohort consisted of 136 patients (median age 62 years, range 22 – 83 years, data not available from 5 patients): 131 liver-, four kidney-, and one heart-transplant recipients. The patients had received the organ transplant more than five years ago (median 10 years, range 5-26 years) and they all received calcineurin inhibitors with declining levels, but remained moderately immunosuppressed throughout the follow-up.

Fresh skin biopsies were taken as 5-mm punch biopsies and rapidly frozen and stored at -70°C until analysis. Healthy skin samples were available from 133 patients, in nearly all cases from the clavicular region. Biopsies from suspected malignant or premalignant (19 carcinoma, 1 melanoma, 4 actinic keratosis) skin were available from 19 patients (1-4 biopsies per patient). Altogether, DNA of 163 skin biopsies were extracted and analyzed. A corresponding serum sample, taken the same day as the skin biopsy, was available from 123 patients. Additionally, of the CuV, BuV or TuV sero- or genopositive patients, 1-3 serum samples of each patient were obtained at various time points before the skin biopsy. In total, 146 serum samples were available from 124 patients.

Immunocompetent healthy adults

Healthy adults (total n=98) with 159 skin biopsies obtained from areas of epicutaneous tests with allergens (n=74) or irritants (n=85) were included as a control group (median age 43 years, range 18–67 years). Two adults had mild psoriasis and 13 had atopic dermatitis, but none had known skin malignancies. Four-mm punch biopsies of skin were obtained from the

backs of all subjects and were then immersed in RNA-later liquid, frozen and stored at -80°C. A corresponding serum sample was available from 78 patients.

Ethics

A written informed consent was obtained from the subjects, and the Ethics Committee of Helsinki and Uusimaa Hospital District or the National Supervisory Authority for Welfare and Health (Valvira) approved the study. The study was conducted in accordance with the relevant guidelines and regulations.

DNA extraction

DNA from FFPE skin samples (1-20 x 10µm sections, depending on the amount of tissue in the blocks) was extracted with QIAamp DNA FFPE Tissue Kit (Qiagen) with some modifications to the manufacturer's instruction (see Supplementary information). DNA from freshly frozen skin biopsies (~ 4 to 5 mm diameter) was extracted with the QIAamp DNA Mini Kit or AllPrep DNA/RNA/miRNA Universal Kit (Qiagen) and from sera (200 µl) with QIAamp DNA Blood Mini Kit (Qiagen), according to the manufacturer's instructions. The extracted DNA was eluted in 100 µl AE buffer. All DNA extracts were stored at -20°C.

BuV-TuV-CuV multiplex qPCR

To detect and quantify BuV, TuV, and CuV DNA, a multiplex real-time quantitative PCR was developed with primers and hydrolysis probes located in the NS1 region of BuV [10] and the VP2 regions of TuV and CuV (Supplement and Table S1).

After optimization, the PCR reactions consisted of 1x Maxima probe qPCR Master Mix (Thermo Scientific) without ROX as passive reference dye, 0.5 µM concentration of each primer (except for the CuV fwd primer which was 1.0 µM), 0.2 µM each of BuV and TuV probes, 0.4 µM of CuV probe, 5µL template, and molecular biology-grade H₂O to a final volume of 25 µL. After initial denaturation and enzyme activation at 95°C for 10 min, 45 cycles of amplification consisting of 15s at 95°C and 1 min at 62°C were performed.

All qPCR determinations were done in duplicates with the AriaMx Realtime PCR System (Agilent Technologies) and all positive samples were confirmed by singleplex qPCR, cloned and sequenced. The CuV DNA-positive samples were further re-amplified to obtain longer 583nt- or 230nt-PCR amplicons by using the primers CuV 580 fwd or CuV 230 fwd and CuV rev (Table S1). To avoid contamination, the FFPE-blocks were cut with disposable blades, the samples, master mix components, and plasmid templates were handled in separate rooms and all templates were added in laminar hoods. All runs included molecular biology-grade water as no-template controls and plasmids as positive controls and for generation of standard curves (see Supplementary Material).

All DNA extracts from clinical samples were subjected to the reference gene, *RNase P*, qPCR for quality control and cell-count quantification, as described [11]. Parvoviral quantity is expressed as copies per 10⁶ cells.

BuV1-3-TuV-CuV IgG EIA

A total of 266 serum samples from 42 CTCL and 124 transplant patients as well as 78 immunocompetent adults were analysed by an in-house BuV1-3-TuV-CuV IgG EIA and all samples showing an OD >0.1 were confirmed with competition EIA to block cross-reactive antibody binding, as described [7, 12] and outlined in the Supplement.

Statistical analysis

Statistics were calculated with R, version 3.4.3 (R Development Core Team, Vienna, Austria) for Mann-Whitney U, Pearson's χ^2 , and one-way ANOVA tests.

Results

Multiplex qPCR from tissue biopsies

CuV DNA was detected in the cutaneous lymphoma lesions of 4/25 (16.0%) CTCL patients (Table 1). The patient with the highest CuV DNA quantity (C-53; up to 7x10⁸ copies per one million cells) had also melanoma and prostate cancer (Table 2). We thus studied 12 additional FFPE tissue samples from this patient and found that two sentinel lymph nodes and both non-malignant and malignant (melanoma) skin samples were CuV-DNA positive with variable viral loads. Interestingly, the highest CuV loads were observed in the CTCL and pre-cancerous CTCL skin biopsies, whereas the two prostate cancer samples of this patient and those of patient C-15, were all CuV DNA negative. No BuV or TuV DNAs were found in any of the FFPE tissue biopsies of the 25 CTCL patients.

Of the 136 organ transplant recipients, 4 (2.9%) harbored CuV DNA in skin (Table 1), but the corresponding serum samples were CuV-DNA negative. Two of these 4 patients had also skin cancer (Table 2); one was CuV-DNA positive in both healthy skin and basal cell carcinoma (patient P-065), and the other in healthy skin, pre-malignant skin and squamous cell carcinoma (patient P-A). The two remaining CuV DNA-positive biopsies were of healthy skin (P-022 and P-108, Table 2). No BuV or TuV DNAs were found in any of the 163 skin biopsies from the 136 transplant patients.

All 159 freshly frozen skin samples from the 98 immunocompetent adults were negative for BuV, TuV and CuV DNA (Table 1). Overall, the CuV DNA prevalence in skin was significantly higher in CTCL patients than in either the transplant recipients or healthy controls ($P < .001$; Pearson's χ^2 test). In the three patients with samples available from both malignant and non-

malignant skin (Table 2), the CuV-DNA loads were higher in the malignant skin samples, but the sparse number of samples did not allow valid statistical analysis. The housekeeping-gene qPCR was positive in all samples.

Sequencing of the CuV DNA

All CuV-qPCR products were cloned and confirmed by sequencing to be identical or nearly identical to those in GenBank. The longer CuV amplicon of 583 nts was obtained from six skin tissues of the four transplant patients and from five CuV high-copy-number tissues of CTCL patient C-53 (Table 2). All long CuV sequences amplified from different tissues of the same individual were identical, whereas those from distinct patients varied (Figure 1). Furthermore, the cloned 91-nt amplicon of patient C-26 had 2-3 mismatches compared to the others. All sequences are in GenBank, accession nos. MH822920-30, or in Figure S2.

Protoparvovirus IgG EIAs

Of the 25 CTCL patients with skin biopsies, corresponding serum samples were available from only two, one was CuV-IgG positive and one negative. However, including a cohort of 40 additional CTCL patient sera, 2/42 (4.8%) harbored BuV1 IgG and 4/42 (9.5%) CuV IgG (Table 3). None of the 42 patients showed BuV2, BuV3 or TuV IgG. Unfortunately, no corresponding serum samples were available from the CTCL patients with CuV DNA-positive skin.

The overall IgG seroprevalence in the organ transplant cohort was 2.4% for all bufaviruses (0.8% for each BuV genotype), 0.8% for TuV and 6.5% for CuV (Table 3). The BuV1 IgG-positive patient was also CuV-IgG positive, all others were IgG positive for only a single protoparvovirus. Further, 1-3 serum samples from all seropositive patients (n=11), obtained at different times before the skin biopsy, were analyzed for protoparvovirus IgG (Table 4). Nine of 11 patients were seropositive already in the first sample, which was taken in most cases before or at the time of transplantation. In addition, these patients showed BuV and/or CuV IgG for 5 to >20 years. Besides these nine patients with long-term seropositivity, one patient (P-093) seroconverted for CuV and another (P-055) for TuV during the post-transplant follow up (Table 4). The TuV IgG remained for 10 years, whereas the transient CuV IgG could have passively originated from blood transfusion.

Of the four CuV DNA-positive transplant recipients, serum samples were available from three, and all showed CuV IgG with high OD values (Table 4). In contrast, all five CuV IgG-positive recipients without detectable CuV DNA in skin showed significantly lower CuV IgG values in the corresponding serum sample ($P < .05$ (Mann-Whitney U test)).

None of the healthy adults had BuV1-3 or TuV IgG, whereas CuV IgG at low OD (0.123-0.192, verified by blocking) was observed in 3.8% of the 78 serum samples available from this cohort (Table 3).

Discussion

Thus far, human protoparvovirus DNA has been detected mainly in diarrheal feces and two studies have shown the presence of CuV DNA in altogether four malignant CTCL skin lesions and in one melanoma [1, 6]. Immunosuppressed organ transplant recipients have in general been shown to be at risk for epithelial skin cancers [13]. In this study, we detected by qPCR CuV DNA in skin biopsies of 16.0% of CTCL patients versus 2.9% of organ-transplant recipients but not in any of 98 healthy adults. Both prevalence differences are highly significant ($P < .001$, Pearson's χ^2 test). All samples were BuV and TuV DNA negative. In the original publication of CuV discovery, it was detected by PCR in 2/15 CTCL lesions, whereas 10 skin carcinomas, 16 other skin diseases (8 parapsoriasis, 4 eczema and 4 eczematoid dermatitis), and 3 healthy skin samples were all CuV-DNA negative [1]. Furthermore, one out of 10 melanoma skin samples was reported to harbor CuV DNA [6]. Interestingly, we discovered CuV DNA both in non-malignant and malignant skin of three subjects: two transplant recipients had CuV DNA in basal or squamous cell carcinoma tissue and in healthy skin, whereas one CTCL/MF patient had CuV in both early- and late-stage CTCL lesions, in melanoma, atypical moles, benign skin tumors, and lymph nodes, but not in his prostate samples. This triple-cancer patient harbored CuV DNA in his tissues for at least four years before CTCL diagnosis. Of the overall 215 patients without skin cancer, only two (0.9%) harbored dermal CuV DNA and both were moderately immune suppressed due to transplantation; all other patients with dermal CuV DNA had skin cancer. The difference of dermal CuV-DNA prevalence between cancerous and non-cancerous patients was highly significant [6/44 (14.0%) vs. 2/215 (0.9%), $P < .0001$, Pearson's χ^2 -test].

The classical human parvovirus B19 (B19V) has been shown to persist for life in most solid tissues including skin of healthy immunocompetent individuals [14-17]. Similarly, many animal parvoviruses persist in their host tissues conferring challenges in laboratory and other animal facilities [18-21]. It is thereby possible that CuV does the same. For optimal replication parvoviruses generally need rapidly dividing host cells such as fetal, intestinal, or bone-marrow cells. Some protoparvoviruses therefore show a preference for cancer cells, the oncolytic characteristic of which is also utilized in cancer therapy [22]. While the host-cell tropism of CuV is unknown, the higher prevalence of CuV DNA observed here in cancerous compared to healthy skin could reflect either causality or an incidental finding due to e.g. CuV-positive cell migration or enhanced ability of CuV to replicate in rapidly dividing cancer cells [5, 22].

The fact that the CTCL skin samples, studied as FFPE, showed higher CuV-DNA prevalences than the freshly frozen skin samples of the other subjects, points to a genuine difference in genoprevalence, since PCR is known to be less sensitive in FFPE samples. That all negative controls remained PCR negative as did the BuV and TuV PCRs, further strengthens the credibility of our CuV PCR data. Furthermore, the slight sequence differences seen in the phylogenetic tree of the longer 583nt-CuV sequences amplified from different individuals, also rules against contamination. Identical long CuV sequences were seen only in samples

obtained within the same individual in samples taken up to 4 years apart. Furthermore, sequence differences were identified also in the cloned short amplicon of a low-load FFPE sample.

All 159 non-malignant freshly-frozen skin biopsies of 98 healthy adults were CuV-DNA free. However, the age difference between the healthy adults compared to the other cohorts or that the skin samples were not exactly matched to the corresponding body region as the malignant lesions, could be argued to affect the CuV-DNA prevalence. However, the age profiles of the CTCL and transplant patients were the same, substantiating the significant difference in occurrence of CuV DNA. Of note, the difference in seroprevalence between the CTCL and healthy cohorts did not reach statistical significance. The CuV seroprevalences in these three cohorts concur with our previously observed CuV-IgG prevalences among adults in Finland and globally (0-5.6%) [7].

Our retrospective serological analysis indicate that the transplant recipients maintained CuV-, BuV- or TuV-specific IgG for at least 5-22 years despite immunosuppression. The observed BuV IgG seroprevalences in CTCL, transplant, and healthy adult cohorts (0-4.8%) are in line with our previously published BuV prevalence of 2-3% in Finland [7, 12], but contrasts sharply to the 56% - 85% seroprevalences in the Middle East and Africa [7]. In EIA, BuV2 and CuV antibodies cross-react, which is logical as the VP2s differ at the amino acid level by only 18%. Such CuV-BuV2 cross-reactivity was also observed in our previous study, yet could successfully be blocked by competition [7]. Here as before, dual seropositivity for these two viruses was infrequent. The clinical performance of our competition assay was further augmented by the data from the three patients with both CuV DNA in skin and CuV-specific IgG in serum. However, we also found several CuV-seropositive individuals without viral DNA in the skin, with lower OD values. Notably, our study is the first to correlate CuV DNA and IgG occurrences within individuals and within patient cohorts.

TuV DNA and IgG, on the other hand, have been strikingly rare or absent in any cohort. Only one child has been found TuV IgG reactive, and at low level [12], whereby the current transplant recipient is the second. TuV DNA has not been detected in any clinical material besides the Tunisian child [3]. Thus, more studies are needed to determine whether TuV really is a human virus.

In conclusion, while BuV and TuV DNAs were not found in any tissue samples, CuV DNA was observed in both malignant and non-malignant skin of CTCL and immunosuppressed organ transplant patients but not in healthy skin of immunocompetent adults. Our study reveals a significantly higher CuV-DNA prevalence among the common subtypes of CTCL, compared with transplant recipients and healthy adults. This study further correlates the occurrences of CuV DNA and IgG in individual patients and cohorts. Our results thus suggest that human cutavirus is associated with CTCL. Whether this association can be generalized to all CTCL

subtypes and whether the role in carcinogenesis is causal, consequential, or casual must be determined in future prospective studies.

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Conflicts of Interest

All authors: No conflicts

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Tables and Figures

Table 1. Protoparvovirus genoprevalence in CTCL, transplant, and healthy adult cohorts

Patient				
cohort	n	CuV DNA^a	TuV DNA	BuV DNA
CTCL	25	4 (16.0%)	0 (0%)	0 (0%)
Transplant	136	4 (2.9%)	0 (0%)	0 (0%)
Healthy				
adults	98	0 (0%)	0 (0%)	0 (0%)

^aThe differences of CuV-DNA prevalence between CTCL and transplant recipients as well as CTCL and healthy adults were statistically highly significant ($P < .0001$) by Pearson's χ^2 test.

Table 2. CuV DNA-positive patients. Patient and sample information and CuV-DNA quantity in the sample.

Cohort	Patient	Age, y/ gender	Sampling site	Sample taken	Mean CuV DNA quantity per 1E+06 cells	Transplant/CTCL info; other diseases
Transplant	P-022	77 / male	Healthy skin Serum	11.1.2013 11.1.2013	4.51E+03 ^a Neg	Liver transplanted 5.1.2003
			Healthy skin	29.10.2013	3.92E+01 ^a	
Transplant	P-065	68 / male	Basal cell carcinoma	29.10.2013	3.35E+03 ^a	Liver transplanted 2.12.1991 Diseased 2015
			Serum	29.10.2013	Neg	
Transplant	P-108	57 / female	Healthy skin	16.12.2014	2.71E+03 ^a	Liver transplanted 2.10.2009
			Serum	30.1.2015	Neg	
Transplant	P-A	81 / male	Healthy skin	unknown	2.54E+02	Kidney transplanted 26.8.1986 and 25.6.1997
			Pre-malignant (carcinoma in situ)		2.28E+03 ^a	
			Squamous cell carcinoma		1.00E+04 ^a	
CTCL	C-81	57 / male	CTCL lesion	2016	1.07E+04 ^b	CTCL/Sezary diagnosed 2016; Severe atopic eczema, erythroderma
CTCL	C-26	32 / male	CTCL lesion	2016	5.83E+02 ^c	CTCL/MF diagnosed 2016
CTCL	C-15	83 / male	CTCL lesion	2016	2.64E+01	CTCL/MF diagnosed 2016; Prostate cancer
			Prostate A	2012	Neg	
			Prostate B	2015	Neg	
CTCL	C-53	63 / male	CTCL lesion A	2015	3.35E+07 ^a	CTCL/MF diagnosed 2015; Melanoma (no metastases), prostate cancer
			Prostate, apex	2012	Neg	
			Prostate, basis	2012	Neg	
			Lymph node (sentinel to prostate)	2012	3.06E+03	
			Melanoma A	2013	7.18E+02	
			Melanoma B	2013	2.34E+02	
			Seborrheic keratosis	2013	6.91E+05	
			Lymph node (sentinel to melanoma)	2014	1.07E+05	
			Lymphocytic atypia (early CTCL)	2015	1.21E+08 ^a	
			Dysplastic nevus A	2015	5.54E+06 ^a	
			Dysplastic nevus B	2015	4.84E+06 ^a	
			CTCL lesion B	2015	7.26E+08 ^a	
			Dermatofibroma	2015	1.65E+05	

Neg, negative; MF, mycosis fungoides.

^aLonger 583-nt amplicons were sequenced of these tissues (Figure 1); ^bThis sample was also PCR positive with the primers CuV 230-fwd and CuV rev (Table S1); ^cThis 91-nt amplicon was 2-3 nt dissimilar to all other amplicons (Figure S2); ^dIn chronological order.

Table 3. Protoparvovirus seroprevalence in transplant, CTCL and healthy adult cohorts

Patient cohort	n	IgG towards any BuV	BuV genotype-specific IgG			TuV IgG	CuV IgG
			BuV1	BuV2	BuV3		
CTCL	42	2 (4.8%)	2 (4.8%)	0 (0%)	0 (0%)	0 (0%)	4 (9.5%)
Transplant	124	3 (2.4%)	1 (0.8%) ^a	1 (0.8%)	1 (0.8%)	1 (0.8%)	8 (6.5%)^a
Healthy adults	78	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	3 (3.8%)

^aone patient had both BuV1 and CuV IgG.

The differences in seroprevalence did not reach statistical significance (Pearson's χ^2 test).

Table 4. Longitudinal IgG EIA results (OD values) of BuV, TuV, or CuV IgG-positive transplant patients. All reactions showing OD>0.1 were confirmed with competition assay. Competition-confirmed IgG EIA results are marked with bold.

Patient	Transplant date	Sampling date	CuV DNA in skin	Confirmed IgG EIA result	BuV1	BuV2	BuV3	TuV	CuV
P-022	5.1.2003	5.1.2003	Pos	CuV	0.031	0.881	0.020	0.027	1.448
		28.1.2003		CuV	0.016	0.254	0.015	0.011	0.554
		11.1.2013		CuV	0.008	1.262	0.008	0.007	2.165
P-065	2.12.1991	1.12.1991	Pos	CuV	0.025	1.070	0.026	0.029	3.135
		30.12.1991		CuV	0.008	0.204	0.017	0.016	0.805
		29.10.2013		CuV	0.023	0.435	0.024	0.021	1.477
P-108	2.10.2009	30.6.2009	Pos	CuV	0.042	1.451	0.032	0.044	1.978
		16.12.2014		serum not available					
		30.1.2015		CuV	0.019	0.579	0.019	0.026	1.241
P-018	21.11.2003	21.11.2003	Neg	BuV1 and CuV	2.066	0.132	0.050	0.135	0.352
		31.12.2012		BuV1 and CuV	0.578	0.056	0.028	0.046	0.155
P-012	14.11.2002	3.11.2002	Neg	CuV	0.025	0.140	0.022	0.025	0.415
		1.12.2002		CuV	0.033	0.095	0.026	0.025	0.336
		3.12.2012		CuV	0.018	0.036	0.011	0.012	0.117
P-037	7.3.2001	7.3.2001	Neg	CuV	0.038	0.420	0.042	0.090	1.256
		27.3.2001		CuV	0.039	0.370	0.049	0.056	0.830
		13.3.2013		CuV	0.062	0.282	0.055	0.121	0.829
P-060	10.4.1994	27.11.2002	Neg	CuV	0.026	0.285	0.027	0.024	0.815
		9.10.2013		CuV	0.053	0.267	0.040	0.036	0.660
P-093	1st tx 19.3.1997, 2nd tx: 26.8.1998	14.4.1997	Neg	Neg	0.022	0.027	0.015	0.017	0.028
		25.8.1998		Neg	0.014	0.025	0.012	0.028	0.044
		23.9.1998		Neg	0.019	0.026	0.022	0.019	0.030
		12.3.2014		CuV	0.034	0.143	0.023	0.021	0.294
P-034	11.3.2007	20.12.2006	Neg	BuV2	0.064	0.169	0.037	0.044	0.067
		21.4.2007		Neg	0.025	0.085	0.028	0.035	0.060
		5.3.2013		BuV2	0.038	0.168	0.022	0.025	0.033
P-070	4.4.2002	23.2.2002	Neg	BuV3	0.015	0.018	0.333	0.016	0.024
		17.5.2002		BuV3	0.014	0.014	0.120	0.015	0.023
		12.11.2013		BuV3	0.020	0.014	0.229	0.019	0.015
P-055	12.8.2001	15.8.2001	Neg	CuV	0.042	0.053	0.045	0.057	0.232
		28.2.2003		TuV	0.017	0.048	0.026	0.342	0.019
		24.9.2013		TuV	0.044	0.068	0.035	0.367	0.030

Pos, positive; Neg, negative; tx, transplant.

The difference in CuV OD values of corresponding serum samples from CuV DNA-positive and -negative recipients was statistically significant, $P < .05$ (Mann-Whitney U test).

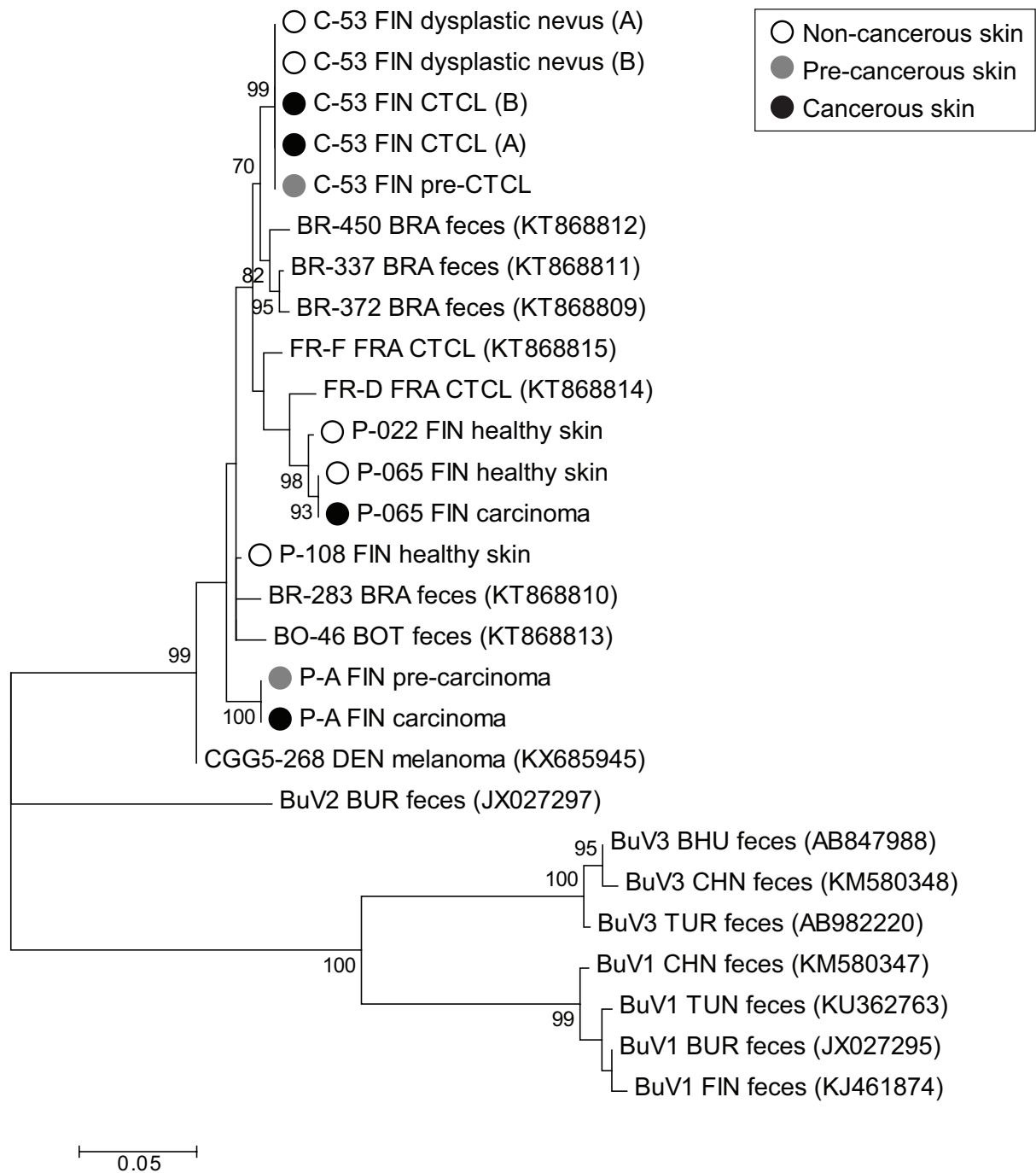


Figure 1. Maximum likelihood phylogenetic tree made with 547-nt amplicons of CuV and BuV isolated from this study and worldwide. The tree was constructed with MEGA7, using the gamma distributed general time reverse model (GTR +G) with 1000 bootstrap resampling. The bootstrap values (≥ 70) are shown at nodes. Circles indicate isolates from this study. Name of each sequence indicates patient or virus strain number, the country of origin and sample type with existing GenBank accession numbers in the parentheses. Abbreviations: BHU, Bhutan; BOT, Botswana; BRA, Brazil; BUR, Burkina Faso; CHN, China; DEN, Denmark; FIN, Finland; FRA, France; TUN, Tunisia; TUR, Turkey. GenBank accession numbers of sequences in this study are MH822920-30.